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Research report

Immunocytochemical localization of AMPA receptors in the rat inferior colliculus

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Abstract

Immunocytochemistry was used to study the distribution of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subtypes in the inferior colliculus (IC) of genetically epilepsy-prone rats (GEPR-9s) and normal Sprague–Dawley (SD) rats. The analysis was conducted using 3 antibodies specific for glutamate receptor subtypes, GluR 1, GluR 2/3, and GluR 4. Light microscopy showed that immunostaining of the IC was most dense with the GluR 2/3 antibody for both strains of animals. The amount of GluR 2/3 immunolabeling was similar for sound-stimulated GEPR-9s, seizure-naïve GEPR-9s, and SD rats. The electron microscopy of GluR 2/3 in the IC revealed immunoreaction products associated with the postsynaptic densities of asymmetric synapses. The thin sections had comparable amounts of reaction product in dendrites or dendritic spines for both strains. Since the distribution and quantity of AMPA receptors in the IC of GEPR-9s and SD rats are similar, our results indicate that altered AMPA receptors are probably not the primary cause of seizure initiation in GEPR-9s. © 1997 Elsevier Science B.V.

Keywords: Immunocytochemistry; Genetically epilepsy prone rat; Seizure; Epilepsy; Glutamate receptor

1. Introduction

Glutamate is acknowledged as a major excitatory neurotransmitter in the central nervous system. Its receptor-mediated activity is involved in fast excitatory synaptic transmission [6], neurotransmitter release [1] and, at toxic levels, ischemia [3] and epilepsy [5]. Ionotropic glutamate receptor subtypes have been characterized as being either *N*-methyl-D-aspartate (NMDA), kainic acid (KA), or α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). AMPA receptors have been further divided into GluR 1, GluR 2, GluR 3, and GluR 4 subtypes [16] and are distributed throughout most brain regions [20]. Petralia and Wenthold [26] utilized specific antibodies and immunocytochemical methods to localize AMPA receptors in various brain regions at the light microscopic level. In addition, they examined the localization of AMPA receptors in the hippocampus and cerebellum at the electron microscopic (EM) level.

The inferior colliculus (IC) is particularly important in the auditory pathway because it is a major relay center for processing of sound. Faingold et al. [12] found that excita-

tory amino acids, such as glutamate, increased the spontaneous firing of neurons in the central nucleus of the IC (CNIC). Thus, glutamate plays an important role in sound-evoked excitation of the CNIC. Other studies examined the effects of chemically manipulating the amount of excitation and inhibition in the IC. For example, microinjections of glutamate agonists in the CNIC generated audiogenic seizures in normal rats [22].

The CNIC of genetically epilepsy-prone rats (GEPR-9s) is considered to be the brain region responsible for the initiation of sound-stimulated clonic–tonic seizures [14]. Although several studies proposed mechanisms for seizure initiation in the CNIC, the exact process is still unknown. Currently, sound stimuli are transmitted from the cochlear nuclei, into the lateral lemniscus and then to the CNIC [2]. Lesion studies have shown that the connections between the central and external nuclei of the IC are essential for audiogenic seizure response [28]. Furthermore, recent data that examined the expression of *c-fos* following seizures in GEPR-9s confirm the role of the pericentral nuclei of the IC and the deep layers of the superior colliculus in seizure propagation [30]. At the cellular level, the CNIC of GEPR-9s was shown to contain a number of physiological and biochemical abnormalities, including increased neuronal

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firing [11] and increased amounts of glutamate [31]. Microinjections of glutamate agonists into the IC increased the audiogenic seizure susceptibility of GEPR-9s [22]. Blocking glutamate synthesis in the IC prevented the onset of audiogenic seizures [15]. In a review article, Seeburg [33] hypothesized that increased excitation mediated by glutamate may be responsible for the onset of seizures in several models. Since the analysis of glutamate receptors will provide new data about glutamate's function in the IC, we used light microscopic immunocytochemistry to localize GluR 1, 2/3, and 4 in the rat IC. In addition, the GluR 2/3 subunit was localized in the CNIC at the EM level. To assess whether GEPR-9s display an abnormal distribution of AMPA receptors, we have examined the localization of these receptors in the IC of GEPR-9s and compared it to that in normal rats.

2. Materials and methods

Thirteen adult Sprague–Dawley (SD) rats (250–400 g) were deeply anesthetized with an intraperitoneal injection of Nembutal (0.15 ml/100 g b.wt.). Hindleg and tail-pinch tests were implemented before the chest cavity was opened for the intracardial perfusion using a previously described protocol for the localization of glutamate receptors [26]. Initially, approximately 200 ml of 0.12 M phosphate buffer, pH 7.3 was used to clear the blood from the circulatory system. Then 550 ml of ice-cold 4% paraformaldehyde and 0.1% glutaraldehyde fixative (pH 7.4) in 0.12 M phosphate buffer was perfused. Brains were removed from the skulls and kept in fixative at 4°C overnight, then switched to phosphate-buffered saline (PBS) the following day.

In addition to the normal SD rats, 13 GEPR-9s (250–400 g) were used in this study and kept under normal conditions. GEPR-9s have the most severe audiogenic seizures in this strain (see review [29]), and were obtained from Dr. Phillip Jobe at the University of Illinois, College of Medicine, Peoria, IL. Both GEPR-9s and SD rats were tested for audiogenic seizures using an isolated padded chamber. An electrical bell (105 dB) was rung for 3–10 s or until the onset of a seizure. All GEPR-9s displayed maximal convulsions of score 9 [18], characterized by wild running and hind-leg extensions. The SD rats used in the study received an audiogenic response score of 0.

To determine whether glutamate receptors are influenced by seizure activity, we also analyzed a group of seizure-naïve GEPR-9s ($n = 9$). These animals had no exposure to ringing bells and did not experience an audiogenic seizure in the laboratory.

2.1. Immunocytochemistry

Coronal sections through the rostrocaudal extent of the IC [24] were obtained with a Vibratome at a thickness of 50 μ m. Selected sections from SD rats, GEPR-9s with

audiogenic seizures and naïve GEPR-9s were incubated at room temperature in 10% normal goat serum (Vectastain) in PBS for 1 h, then incubated overnight in one of three primary antibodies in PBS at 4°C. Polyclonal rabbit antibodies against glutamate receptors 1, 2/3, and 4 were used at the following concentrations: 2.4 μ g/ml for GluR 1, 2.0 μ g/ml for GluR 2/3 and 1.2 μ g/ml for GluR 4 [26]. The antibodies were obtained from Chemicon (Temecula, CA) and were affinity-purified using immobilized peptides of either the original peptide or a segment of the peptide as an antigen. Each antibody was specific for the GluR subtype and was characterized by the immunoabsorption of detergent solubilized [3 H]AMPA binding activity [35].

After extensive washing in PBS, the sections were further processed according to the ABC technique [17] using a Vectastain Kit (Vector Laboratories, Burlingame, CA). To localize sites of antigen–antibody binding, sections were treated with diaminobenzidine (DAB) (10 mg/20 ml PBS and 5 μ l of 30% hydrogen peroxide) for 15 min. After two final washes of PBS, the sections for light microscopy were mounted on gel-coated slides. Slides were left in a slide warmer overnight to completely dry and were later soaked in an ascending series of ethanols at 5-min intervals, followed by xylene. Cover slips were immediately attached with DPX mounting medium.

Sections for electron microscopy were osmicated with cold (4°C) 1% OsO₄ for 30 min, dehydrated in graded ethanol, and embedded in Medcast. Semithin 2 μ m sections were first obtained from blocks containing the CNIC before ultrathin sections were cut, collected on Formvar-coated slot grids, stained with lead citrate and uranyl acetate and examined with a Philips CM-10 electron microscope.

3. Results

3.1. Light microscopic localization of AMPA receptors

The immunocytochemical localization of GluR 1, GluR 2/3, and GluR 4 in the IC was consistent with the light microscopic data of Petralia and Wenthold [26]. Immunostaining for GluR 1 and GluR 4 was light and homogeneous throughout all subnuclei of the IC in SD rats, including the central nucleus, dorsal cortex, and external nucleus (Fig. 1). Only a small number of neurons in the IC displayed any immunolabeling for GluR 1 or GluR 4 (not shown). In contrast, sections of the IC that were immunostained for GluR 2/3 were more darkly labeled than those incubated with GluR 1 and GluR 4 antibody. Many somata in the IC were immunolabeled for GluR 2/3, and they displayed different sizes. The central nucleus contained a majority of small and medium-sized GluR 2/3 immunolabeled cells. The dorsal nucleus and the dorsal areas of the central nucleus contained smaller labeled neurons compared to the larger GluR 2/3-positive neurons in the

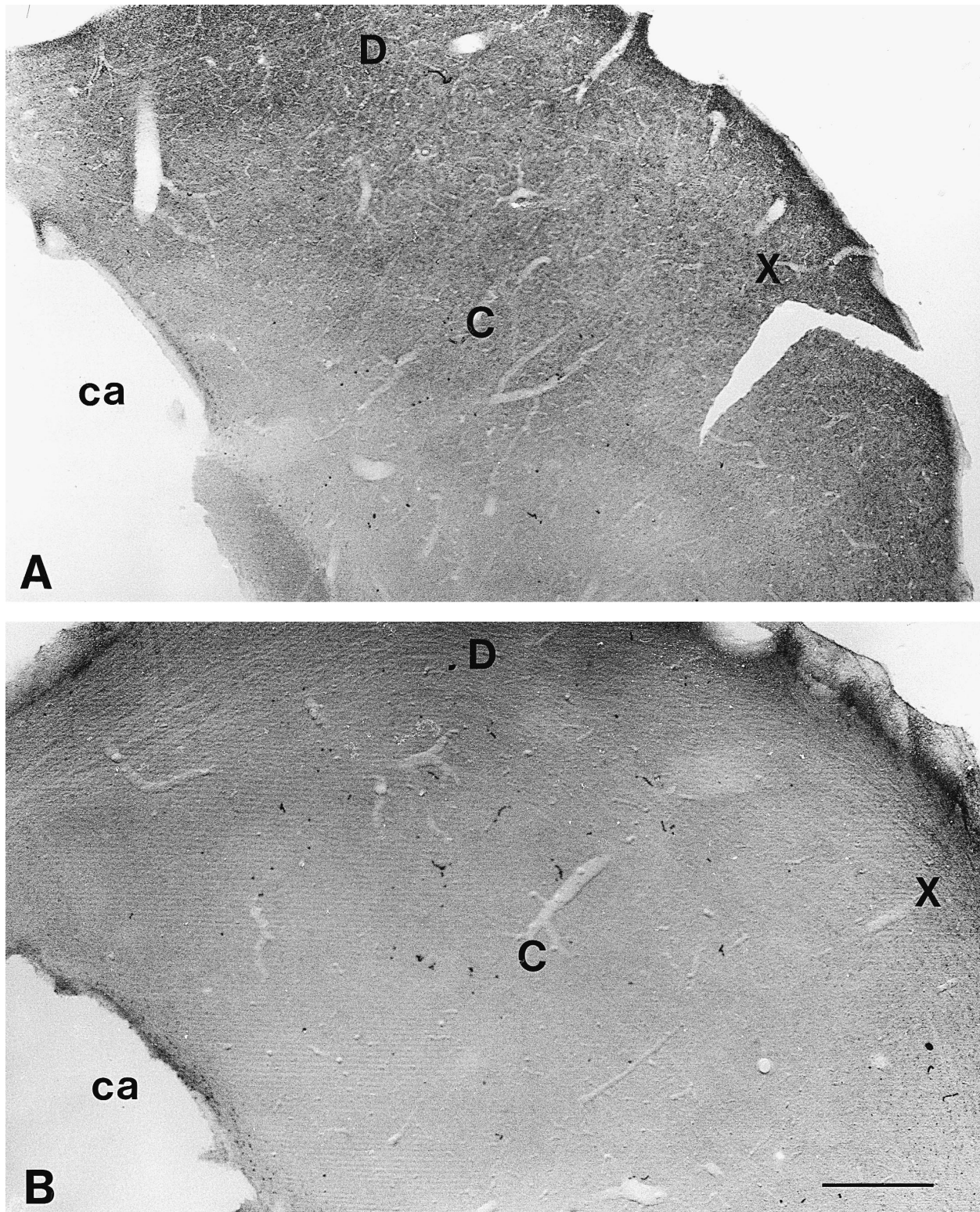


Fig. 1. Low magnification light micrographs of coronal sections of the CNIC in SD rats. Immunostaining is light and homogeneous throughout the section for GluR 1 (A) and GluR 4 (B). C, central nucleus; D, dorsal cortex; X, external nucleus; ca, cerebral aqueduct. Scale bar = 0.3 mm.

external nucleus and ventral areas of the central nucleus (Fig. 2A). Several of the larger neurons also had immunolabeled proximal dendrites. However, the dendritic staining was not adequate to distinguish the dendritic arbors of

stellate and disk-shaped CNIC neurons [23]. Control sections which were not incubated with primary antibody to AMPA receptors were unstained.

Seizure-naïve and sound-stimulated GEPR-9s displayed

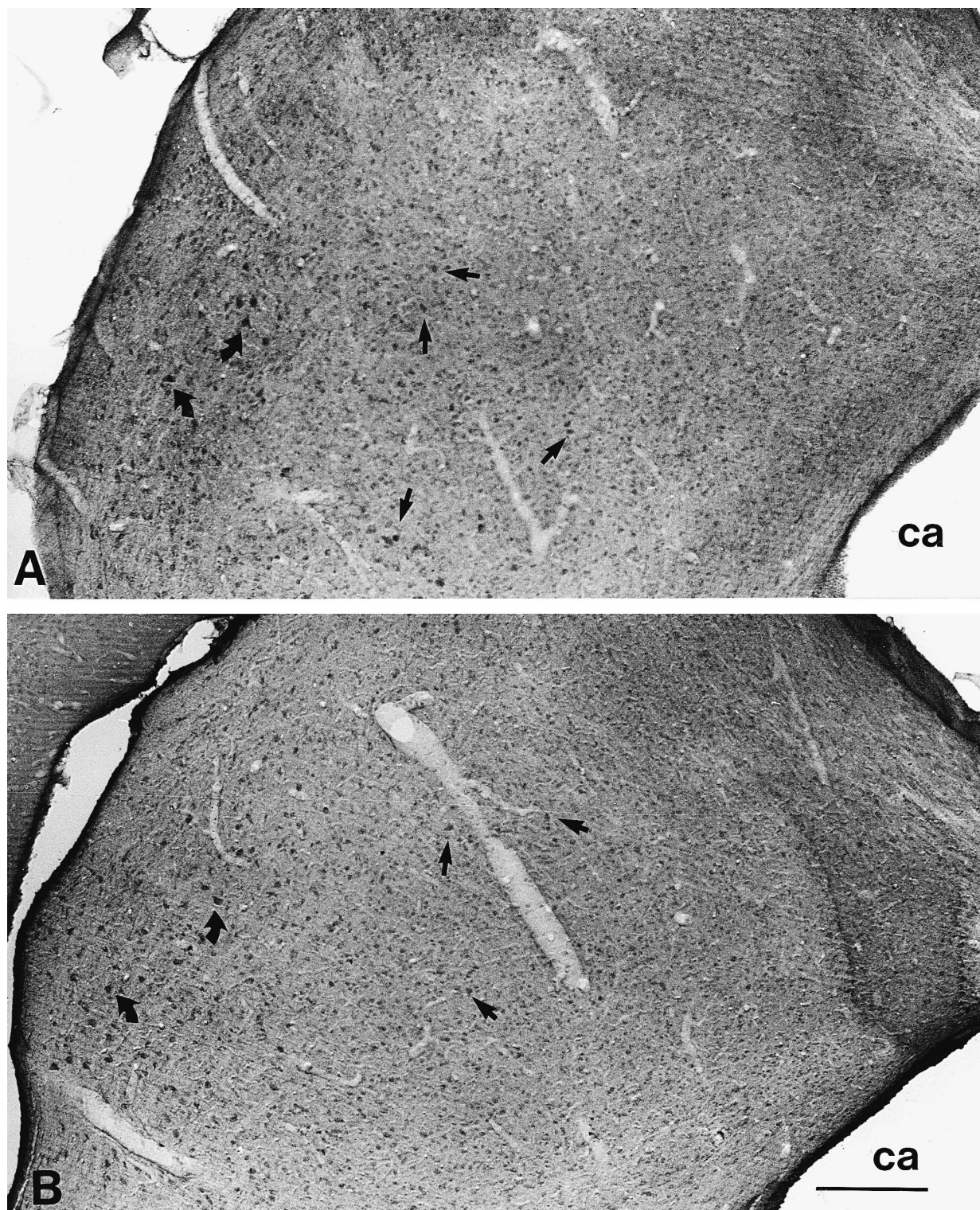


Fig. 2. Low magnification light micrographs of coronal sections through the IC immunolabeled with antibodies against GluR 2/3 in SD rats (A) and stimulated GEPR-9s (B). Many immunostained neurons (arrows) are found in the CNIC. The neurons in the external nucleus (curved arrows) appear larger. ca, cerebral aqueduct. Scale bar = 0.3 mm.

similar immunostaining patterns for all AMPA receptors examined (Fig. 3). Sections immunolabeled for GluR 1 and GluR 4 displayed light staining as observed in SD rats. The immunostained neurons for GluR 2/3 were more

densely stained and were labeled throughout the IC (Fig. 2). This immunolabeling pattern for AMPA receptors in the IC of GEPR-9s was similar to that of the SD rats. Immunolabeling for GluR 2/3 in GEPR-9s was present in

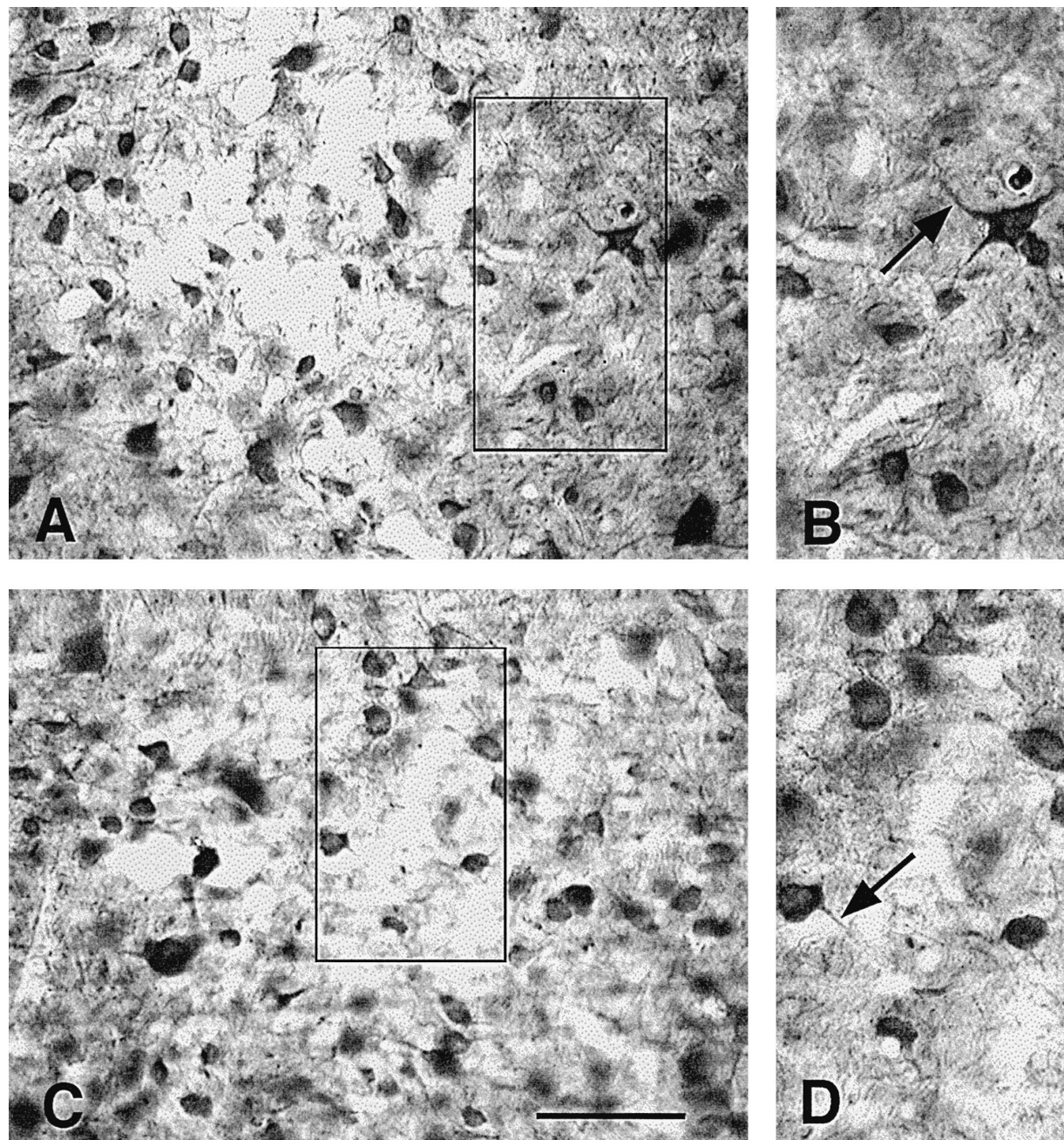


Fig. 3. Enlargements of coronal sections through the IC immunolabeled with GluR 2/3 in seizure-naive (A,B), and sound-stimulated (C,D) GEPR-9s. Small-, medium-, and large-sized neurons were immunolabeled in both. Boxed regions in A and C are enlarged in B and D, respectively. Proximal dendrites (arrows) of larger neurons also contain immunoreaction product. Scale bars: (for A,C) = 100 μ m; (for B,D) = 200 μ m.

small-, medium-, and large-sized neurons. Some of the proximal dendrites of the larger neurons were also immunolabeled (Fig. 3).

3.2. Electron microscopic localization of GluR 2/3 receptors

The ultrastructural distribution of immunolabeling for GluR 2/3 was studied in the ventral lateral portion of the CNIC. Immunostaining for GluR 2/3 in this region of SD

rats was associated with the postsynaptic densities of asymmetric synapses. These synapses were found on somata, dendrites, and dendritic spines (Fig. 4A,B). The synaptic vesicles in axon terminals of these labeled synapses were mostly round. Asymmetric synapses were characterized by a wider synaptic cleft and a thicker postsynaptic membrane than symmetric synapses [25]. Electron micrographs also showed the presence of asymmetric synapses that had little or no reaction product at their postsynaptic densities. Furthermore, the symmetric

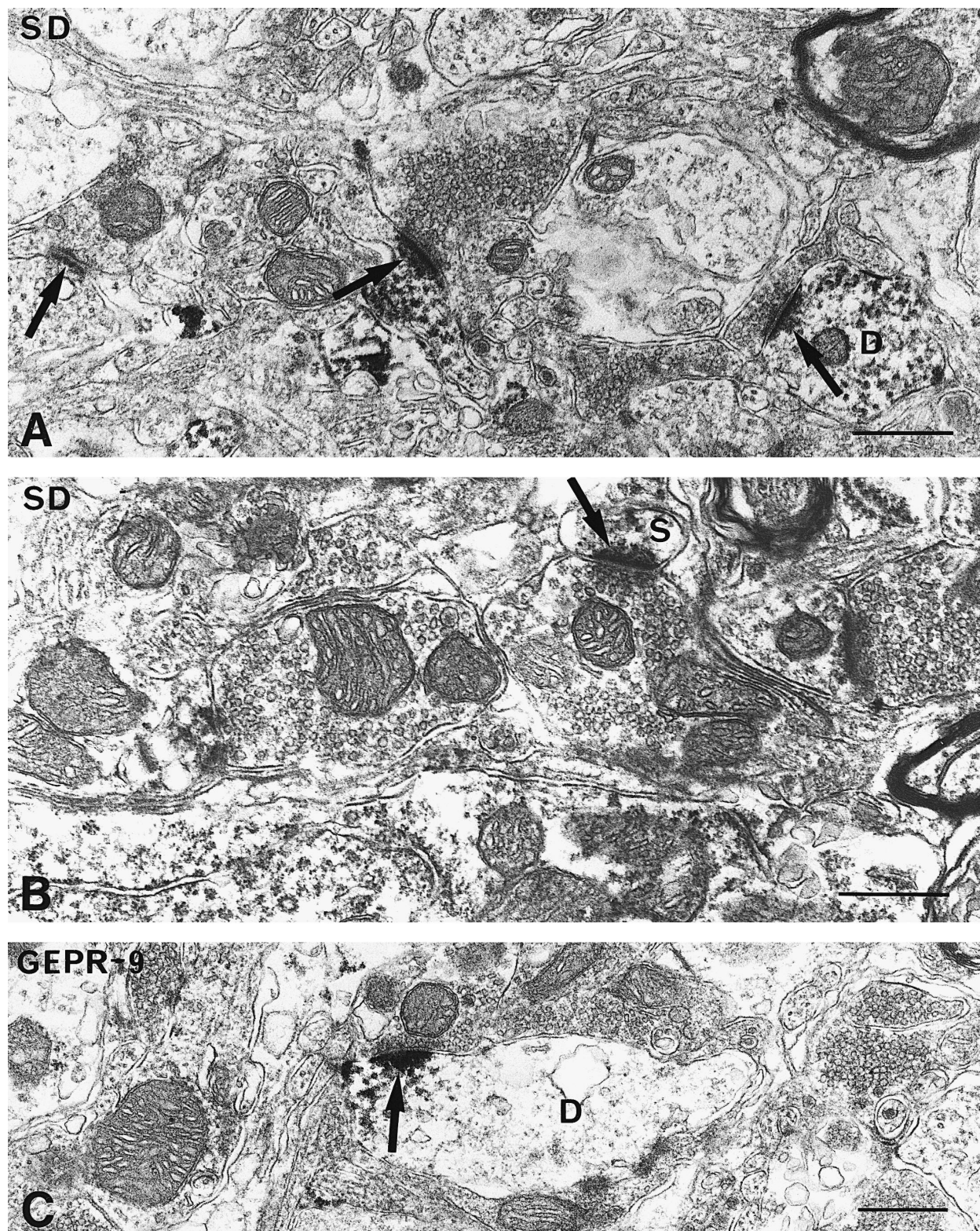


Fig. 4. Electron micrographs of the CNIC immunolabeled with antibodies against GluR 2/3. Labeling is associated with the postsynaptic densities of asymmetric synapses characterized by round synaptic vesicles and thicker postsynaptic membranes. Immunoreaction product (arrows) is localized at axodendritic (A) and axospinous (B) synapses in SD rats and at axodendritic (C) synapses in GEPR-9s. D, dendrites; S, spine. Scale bars = 0.5 μ m.

synapses in the CNIC did not display immunostaining associated with their postsynaptic densities. Dendrites that were postsynaptic to several axon terminals showed im-

munolabeling that was preferentially located adjacent to postsynaptic densities.

The same pattern of immunostaining for GluR 2/3 was

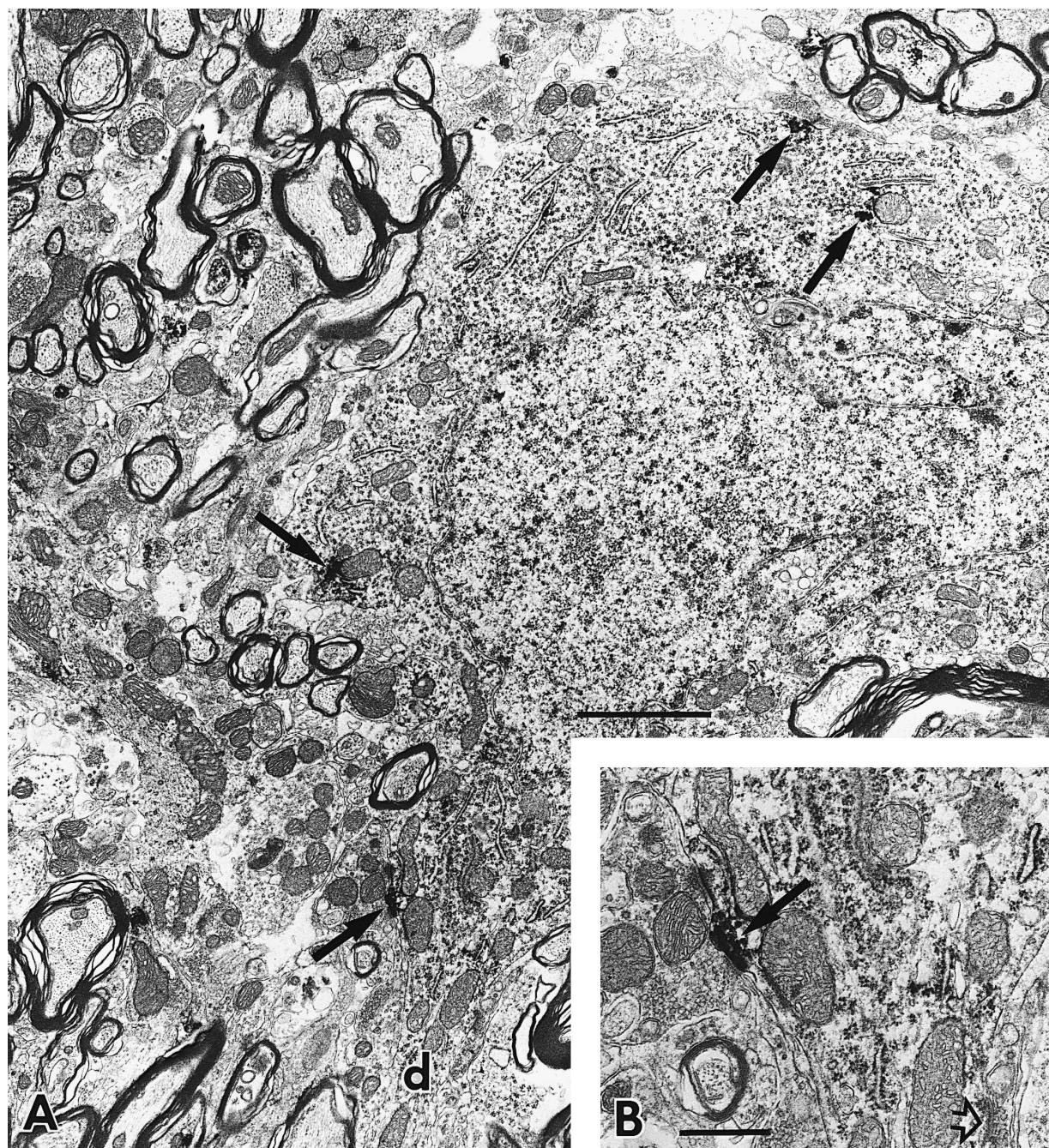


Fig. 5. A: Low magnification electron micrograph of somatic immunolabeling of GluR 2/3 in the GEPR-9s. Arrows indicate areas of immunoreaction product throughout the soma and into a dendrite (d). B: Enlargement of axodendritic synapses showing proximal dendrite (d) and selective immunolabeling at the asymmetric synapse (arrow), but not at the symmetric one (open arrow). Scale bars: (in A) = 2.0 μm ; (in B) = 0.5 μm .

observed in sound-stimulated GEPR-9s in that immunostaining was associated with axosomatic, axodendritic and axospinous synapses (Fig. 4C and Fig. 5). Asymmetric synapses displayed immunoreaction product that was associated with postsynaptic densities (Fig. 4C), and the amount of immunolabeling was similar to that observed in SD rats. In addition, GEPR-9s lacked immunolabeling at symmetric synapses (Fig. 5B), and this was also similar to that for the SD rats.

4. Discussion

The immunocytochemical analysis of GluR subunits in the IC demonstrated significant differences in the quantity and distribution of the various receptor isoforms. A major finding in this study was that GluR 2/3 is the predominant receptor in the CNIC based upon its greater immunostaining as compared to that for GluR 1 and GluR 4. Thus, we observed the most dense immunostaining with the

GluR 2/3 antibody in the IC; this finding was consistent with the light microscopic results of Petralia and Wenthold [26]. The low amount of immunolabeling for GluR 1 and GluR 4 probably indicates that these receptor subunits are not expressed as much in the CNIC as GluR 2/3. Another major finding of this study was that GluR 2/3 immunolabeling in the CNIC at the electron microscopic level was associated with the postsynaptic densities of asymmetric synapses. This result is similar to previous findings reported for the hippocampus and cerebellum where GluR 2/3 immunolabeling was also shown to be associated with the postsynaptic densities of asymmetric synapses [26].

The present data show that the immunostaining for GluR receptors in the CNIC of GEPR-9s was similar to that found in SD rats. In addition, both seizure-naïve and sound-stimulated GEPR-9s had the same pattern of immunostaining. These findings suggest that AMPA receptor expression: (1) is probably not different in GEPR-9s; and (2) does not change as a result of seizure activity in GEPR-9s (Fig. 3). These conclusions appear to be contradictory to a role for glutamate in the initiation of audiogenic seizures. However, previous data showed that the CNIC had increased amounts of glutamate in both GEPR-9s and sound-induced epileptic mice [27,34]. Thus, the similarity in immunolabeling for GluR 2/3 between GEPR-9s and SD rats may suggest that a glutamate receptor other than AMPA is involved in mediating excitation for seizure initiation. Indeed, blocking NMDA receptors by both competitive and non-competitive antagonists had anticonvulsive effects in GEPR-9s [7,8]. Consistent with this involvement of the NMDA receptors is the study of Marianowski et al. [19] who found that audiogenic epileptic mice had higher levels of NMDA receptors in the IC compared to normal mice. Anticonvulsant drugs were also used as a means of showing the involvement of both NMDA and AMPA receptors in other audiogenic epileptic mice [9]. Therefore, it is likely that the initiation of seizures in GEPR-9s may involve the NMDA receptor more than the AMPA receptor.

If audiogenic seizures are initiated as a result of an increase in released glutamate, the question of the neurotoxic effects of glutamate would remain. Meldrum and Garthwaite [21] have stated that during normal excitation, the synapse is flooded with a high concentration of glutamate only for a short period of time. Furthermore, glutamate has a rapid dissociation rate from AMPA receptors [4]. Significantly higher concentrations are needed in order to cause cell death. It is possible that constantly repeating audiogenic seizures would lead to excitotoxicity due to glutamate, but since our experimental GEPR-9s received only a minimal amount of audiogenic seizures (1–2), we did not see any evidence of cell death. Consistent with this failure of cell death after seizures in GEPR-9s are data that show paradoxical increases in the total number of neurons and GABA neurons in the CNIC [32].

A number of hypotheses have been proposed to explain

possible mechanisms involved in the seizure activity of GEPR-9s. Recently, Ribak and Morin [29] reviewed the three most commonly accepted views. First, GABA uptake mechanisms in GEPR-9s may be functioning at a faster than normal rate, causing less GABA to be effective in the synapse [10]. Secondly, the neurotransmitter–receptor complex for GABA may be desensitized, thus making inhibition less effective [13]. Lastly, overexcitation may be caused by the reported increased levels of glutamate in the IC [27,34]. Future studies should assess whether this increased glutamate is binding differentially to AMPA or NMDA receptors in GEPR-9s as compared to SD rats.

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